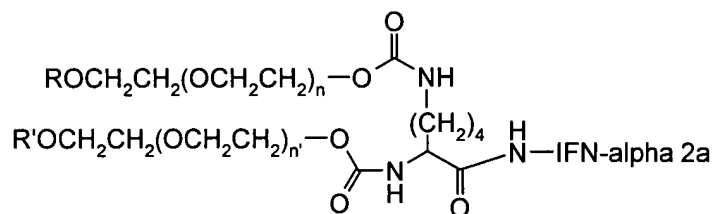


POSITIONAL ISOMERS OF PEGYLATED ALPHA INTERFERON

Background of the Invention

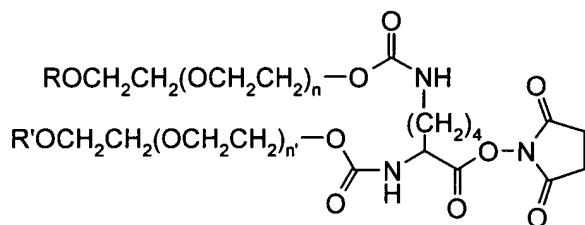
[0001] Interferon alpha-2a plays an important role for the treatment of chronic hepatitis C, but it is limited in its efficacy by the short *in vivo* half-life. To improve the half-life and efficacy, interferon alpha-2a was conjugated with a polyethylene glycol moiety. Pegylation changes physicochemical and biological properties of the protein. One effect is the decrease of the proteolytic degradation and the renal clearance. This increases the half-life of the pegylated protein in blood. Another effect is the altered distribution in the body, depending on the size of the PEG moiety of the protein. Interferon alpha 2a pegylated with a large polyethylene glycol moiety (PEG moiety) such as a 40 kDa branched polyethylene moiety



[0002] wherein R and R' are independently lower alkyl; n and n' are integers having a sum of from 600 to 1500; and the average molecular weight of the polyethylene glycol units in said conjugate is from about 26,000 daltons to about 66,000 daltons;

has an improved biological activity and exhibits sustained adsorption and reduced renal clearance, resulting in a strong antiviral pressure throughout a once-weekly dosing schedule, see Perry M. C., et al. *Drugs*, 2001, 15, 2263-2288 and Lamb M. W., et al. *The Annals of Pharmacotherapy*. 2002, 36, 933-938.

[0003] The method for the pegylation of interferon alpha-2a is described in EP A 809 996. Since this pegylation is performed by reaction of PEG2-NHS



[0004] with primary amino groups on for example lysine or to the N-terminus of the interferon alpha one or more PEG moieties may be attached and form a mixture of unpegylated, mono- and multiple-pegylated interferon. Monopegylated interferon alpha can be isolated from the mixture by methods known in the art. Furthermore, since interferon alpha-2a molecule exhibits 12 sites for pegylation (11 lysines and the N-terminus), such monopegylated interferon is itself a mixture of positional isomers.

Summary of the Invention

[0005] The present invention is concerned with the isolated positional isomers of monopegylated interferon alpha 2a, with a method for their isolation and for their use in the manufacture of medicaments for the treatment of illnesses, especially for the treatment of viral diseases.

Brief Description of the Drawings

[0006] Figure 1 is an analytical IEC-HPLC of 180µg of PEG-IFN alpha 2a showing the separation between positional isomers.

Figure 2 is an SDS-PAGE gel analysis of the positional isomers under non-reduced conditions.

Figure 3 is an SDS-PAGE gel analysis of the positional isomers under reduced conditions.

Figure 4 is a size exclusion (SE-) HPLC used to determine the amount of oligo PEG-IFN forms and aggregates in the different IEC fractions.

Figure 5 is a MALDI-TOF spectrograph showing the molecular weight of each positional isomer.

Figure 6 is a MALDI-TOF Lys-C peptide map of the PEG-IFN reference standard and the peaks 1, 2, 3, 4, 4a, 5, 6, 7, and 8.

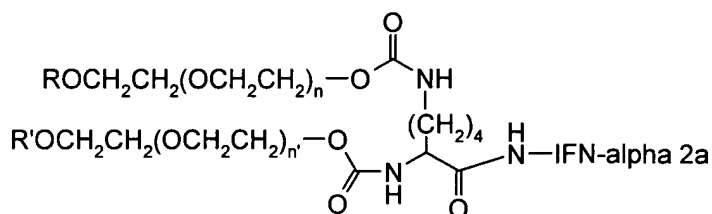
Figure 7 is an RP-HPLC chromatogram of the Lys-C digests of the Peg-IFN reference and peak 4a.

Figure 8 is an analytical HPLC of 5-10µg of PEG-IFN alpha 2a mixture of positional isomers as described in Example 1B.

Figure 9 is a ribbon structure of interferon alpha-2a showing the pegylation sites.

Detailed Description of the Invention

[0007] The present invention provides the positional isomers of pegylated interferon alpha 2a of the formula



[0008] wherein R and R' are independently lower alkyl; n and n' are integers having a sum of from 600 to 1500 and the bond to the IFN-alpha 2a is at one of the lysine residues available on the IFN-alpha 2a polypeptide. The average molecular weight of the polyethylene glycol units in said conjugate is from about 26,000 daltons to about 66,000 daltons, and most preferably is about 40,000 daltons.

[0009] From the possible twelve isomers, nine were isolated and characterized, each of these being conjugated to the branched polyethylene glycol chain at a specific lysine, namely, at Lys(31) to form interferon alpha 2a pegylated at Lys(31) [referred to as PEG-Lys(31)], at Lys(49) to form interferon alpha 2a pegylated at Lys(49) [referred to as PEG-Lys(49)], at Lys(70) to form interferon alpha 2a pegylated at Lys(70) [referred to as PEG-Lys(70)], at Lys(83) to form interferon alpha 2a pegylated at Lys(83) [referred to as PEG-Lys(83)], at Lys(112) to form interferon alpha 2a pegylated at Lys(112) [referred to as PEG-Lys(112)], at Lys(121) to form interferon alpha 2a pegylated at Lys(121) [referred to as PEG-Lys(121)], at Lys(131) to form interferon alpha 2a pegylated at Lys(131) [referred to as PEG-Lys(131)], at Lys(134) to form interferon alpha 2a pegylated at Lys(134) [referred to as PEG-Lys(134)], at Lys(164) to form interferon alpha 2a pegylated at Lys(164) [referred to as PEG-Lys(164)].

[0010] It has been found that PEG-Lys(31) and PEG-Lys(134) have higher activities in an antiviral assay than the mixture, the activity of PEG-Lys(164) was equal to the mixture, whereas the activities of PEG-Lys(49), PEG-Lys(70), PEG-Lys(83), PEG-Lys(112), PEG-Lys(121) and PEG-Lys(131) were lower.

[0011] The invention thus is concerned with new positional isomers of pegylated interferon alpha 2a, namely with PEG-Lys(31), PEG-Lys(49), PEG-Lys(70), PEG-Lys(83), PEG-Lys(112), PEG-Lys(121), PEG-PEG-Lys(131), PEG-Lys(134) and PEG-Lys(164), characterised in that the average molecular weight of the polyethylene glycol moiety (PEG moiety) in said pegylated interferon is from about 26,000 daltons to about 66,000 daltons, especially of about 40000 daltons.

[0012] A chromatography method for the separation of the positional isomers of pegylated interferon alpha 2a based on the local charge differences has been developed. This method consists in a two step separation by ion-exchange chromatography.

[0013] In a further embodiment the invention is thus concerned with a method for the isolation of the positional isomers of pegylated-interferon alpha 2a which consists in the separation of the positional isomers on a preparative liquid chromatography column with a weak-cation exchange matrix; and the further separation and purification of the fractions from the first step on a preparative column, preferably a HPLC column with a strong-cation exchange matrix.

[0014] The separation step a) on the weak-cation exchange matrix was conducted by applying a linear pH gradient from about pH 3,8 to pH 8.0.

[0015] The separation step b) was conducted with linear pH gradient of a sodium acetate buffer (A) to a potassium phosphate buffer (B) starting from an initial pH 4.2 to about 4.6, preferably of about pH 4.4, to a final pH of about pH 6.4 to about 6.8, preferably of pH 6.6, said buffer solutions containing in addition up to 12% ethanol and up to 1.5% diethylene glycol, preferably 10% ethanol and 1% diethylene glycol.

[0016] The elution of the isomers can be influenced by the initial concentration of the buffer solution. The concentration of the buffer solution is from about 3mM to about 15mM sodium acetate, preferably from about 3 to 7mM, ideally from 3.4mM or 6.8mM.

[0017] The separation step b) is carried out at a temperature in the range of about of 27° C to about 35° C, preferably at a temperature of about 30 to 32°C.

[0018] This method can also be used analytically for the analysis of the composition of the positional isomers obtained in the pegylation reaction of interferon alpha 2a.

[0019] The resulting protein samples were collected and analysed by a variety of protein chemical methods such as mass spectrometry peptide mapping, reverse-phase high-performance liquid chromatography (RP-HPLC) peptide mapping, MALDI-TOF spectra of undigested protein, size exclusion HPLC (SE-HPLC) and SDS-PAGE and identified, see examples 2 to 6.

[0020] First, the molecular weight of each isomer was determined by MALDI-TOF spectrometry in order to ensure that the pegylated interferon alpha 2a molecules were still intact after IEC chromatography (Ion Exchange Chromatography) and to confirm the monopegylation. Each IEC peak was measured without further modification. The spectra of all molecules show the expected broad M^+ peaks with maxima at 63 kDa and the corresponding M^{2+} peaks at 32 kDa and M^{3+} peaks at 21 kDa (Fig. 5).

[0021] Second, each isomer was proteolytically digested using endo-Lys-C protease and the resulting MALDI-TOF peptide maps were compared with the one derived from the pegylated-interferon alpha 2a reference standard.

[0022] Interpretation of the spectra and structural identification of the positional isomers is based on the following considerations:

1. Dipegylation of the isomers can be ruled out because of the molecular weight determination of the entire molecule (see above).
2. The single lysine of a specific isomer having the pegylated polymer group attached is not recognised as lysine by the endo-Lys-C protease (2) *New England Journal of Medicine* 2000, 343, 1666-1172. and, therefore, the polypeptide chain is not cleaved at that specific position.
3. It is therefore expected that the peptide map of a specific isomer is lacking the peptides (and only those peptides) which are related to its single pegylated lysine.
4. It is not expected to detect the mass peak of the peptides having the PEG residue attached in the MALDI-TOF peptide maps as the mass range chosen for most accurate detection of the non-pegylated peptides ranges from 850 Da to 6000

Da. The PEG-moiety itself has already a molecular weight of 40 000 Da. However, the pegylated peptides have also been detected using the same digest and trans-3-indoleacrylic acid (IAA) as matrix. For each Lys-C digested isomer a broad peak at 46 - 47kDa was observed, confirming the presence of the monopegylated peptides. Due to the broad mass distribution induced by the PEG-residue, no direct identification of the attached peptides could be made in these experiments (data not shown).

[0023] The resulting peptide maps are shown in Figure 6. Peaks that are missing in comparison to the standard are indicated by arrows.

[0024] Regarding the spectra of the two references of interferon alpha-2a and pegylated-IFN alpha-2a, no significant differences can be seen. Due to the fact that pegylated-interferon alpha 2a is a mixture of different pegylation isomers, all peptide peaks detected for interferon are detected for pegylated-interferon alpha 2a, too.

[0025] In the spectrum of the endo-Lys-C digested protein derived from IEC fraction 1 the peptides comprising amino acids 24 - 31 and 32 - 49 are missing in the region between 850 and 6.000 Da, all other peaks are present. Therefore the PEG residue must be attached to Lys 31.

[0026] The other fractions were identified in the same way. In each case the pegylated peptides are missing in comparison to the reference standard spectrum. For fractions 3 and 4a only one peptide peak is missing, for the second peptide 132 - 133 the mass is too small to be detected in the defined mass window. Only fraction 4a could not be identified with this method, no conclusions could be made.

[0027] In order to identify isomer 4a, an endo-Lys-C peptide mapping method with RP-HPLC/UV detection has been developed. The protein was digested with endoproteinase Lys-C as described for the MALDI-TOF MS peptide mapping. The peptides were separated by means of a water/acetonitrile/TFA (trifluoro acetic acid) gradient.

[0028] With the pegylated-interferon alpha 2a reference standard, 13 peaks were observed. All fractions were collected manually and identified by MALDI-TOF mass spectrometry.

[0029] The assignment of the pegylation site of IEC fraction 4a again was done by comparing the chromatogram of the sample to the one obtained for the reference material. The peak containing the two peptides 134 - 164 and 134 - 165 is clearly missing in the sample chromatogram and therefore IEC fraction 4a can be assigned to the isomer containing the PEG at Lys 164. The chromatograms of the pegylated-interferon alpha 2a reference standard (46 µg/mL) and the one of fraction 4a are shown in Figure 7.

[0030] A graphical representation of the 9 pegylated-interferon alpha 2a positional isomers isolated and characterised is given in Figure 9.

[0031] The in vitro antiviral activity of the isolated isomers was analysed by the protective effect on Madin-Darby bovine kidney (MDBK) cells against the infection by vesicular stomatitis virus (VSV) and compared with a pegylated-interferon alpha 2a standard according to the procedure described in *J. Virol.* 1981, 37, 755-758.

[0032] A further embodiment of the invention is therefore use of positional isomers of pegylated interferon alpha-2a molecule, especially of positional isomers of interferon alpha 2a pegylated at Lys(31), Lys(49), Lys(70), Lys(83), Lys(112), Lys(121), Lys(131), Lys(134) and Lys(164), for the preparation of a medicament for antiproliferative, antiviral and immunomodulatory uses. Especially preferred is the use of interferon alpha 2a pegylated at Lys(31), Lys(134) and Lys(164) for the preparation of such medicaments. The positional isomers can further be used for the preparation of a medicament for the treatment of viral diseases, especially for the treatment of hepatitis C.

[0033] The present invention also comprises the pharmaceutical compositions on the basis of the compounds of formula I or their salts and to methods for producing them.

[0034] The pharmaceutical compositions of the present invention used in the control or prevention of illnesses comprises a positional isomer of pegylated IFN alpha 2a, especially of PEG-Lys(31), PEG-Lys(134) or PEG-(164), more especially of PEG-Lys(31), PEG-Lys(134), and a therapeutically inert, non toxic and therapeutically acceptable carrier material. The pharmaceutical compositions to be used can be formulated and dosed in a fashion consistent with good medical practice taking into consideration the disorder to be treated, the condition

of the individual patient, the site of delivery of the positional isomer of pegylated IFN alpha 2a, the method of administration and other factors known to practitioners.

[0035] Below the methods and material used in the isolation and the characterisation of the positional isomers of pegylated interferon alpha 2a are described in more detail.

[0036] The pegylated interferon alpha 2a (PEG-IFN alpha 2a) used for the isolation of the isomers was produced at Hoffmann-La Roche Inc. by the conjugation of lysine ϵ -amino groups at the surface of the interferon molecule with an activated branched polyethylene glycol moiety of molecular weight 40.000 Da as described in EP A 809996 and in *Bioconjugate Chem.* 2001, 12, 195-202.

[0037] The purity of the samples during the separation of the positional isomers from each purification step was checked using an analytical strong-cation exchange column (TOSOH-BIOSEP, SP-5PW, 10 μ m particle size, 7.5 mm diameter, 7.5 cm length). The column was pre-equilibrated with 3.4 mM sodium acetate, 10% ethanol and 1% diethylene glycol, adjusted to pH 4.4 (buffer A). After loading the PEG-IFN samples, the column was washed with buffer A, followed by an ascending linear gradient to 10 mM dibasic potassium phosphate, 10% ethanol and 1% diethylene glycol, adjusted to pH 6.6 (buffer B). The flow rate was 1.0 mL/min and the detection at 218 nm, the results are given in Figure 1.

[0038] In analogy to the method described above the following analytical method has been found for the analysis of the composition of the positional isomers obtained in pegylation reaction of interferon alpha 2a.

[0039] After separation of the monopegylated interferon alpha from the reaction mixture by methods known in the art, the positional isomers are separated by an analytical liquid HPLC (high pressure liquid chromatography) method on a column charged with a strong-cation exchange matrix such as for example nonporous SP-NPR phase with a particle size of 2.5 μ m from TosoH Bioscience. The mobile phase consist of a buffer A (10% v/v of ethanol; 1% v/v diethylenglycole; 2.3 mM sodium acetate and 5.2 mM acetic acid in purified water; no pH adjustment is made) and a buffer B 10% v/v in ethanol; 1% v/v in diethylenglycole; 16.4 mM KH_2PO_4 ; and 4.4 mM K_2HPO_4 in purified water, no pH adjustment is made), the results are depicted in Figure 8.

[0040] The following examples will further illustrate the invention

Example 1A

Separation of the positional isomers

[0041] A two-step isolation and purification scheme was used to prepare the monopegylated isoforms of PEG-interferon alpha 2a.

[0042] The first step was a separation of the positional isomers on a preparative low pressure liquid chromatography column with a weak-cation exchange matrix (TOSOH-BIOSEP, Toyopearl CM-650S, e.g. Resin Batch no. 82A, the diameter of the column being 16 mm, the length 120 cm). A linear pH-gradient of increasing sodium acetate concentration (25 mM, pH 4.0 up 75 mM to pH 7.8) was applied at a flow rate of 0.7 mL/min. Detection was at 280 nm. With this chromatographic step species 1, 2, 5, 6 and a mixture of 3, 4, 4a, 7 and 8 could be collected, see Table 1.

[0043] The fractions were further separated and purified in the second preparation step. A preparative column with the same matrix as the analytical strong-cation exchange column (Resin Batch no. 82A having a ion exchange capacity of 123 mEq/ml) as described above but larger dimensions (30 mm i.d. and 70 mm length), further a higher flow rate and an extended run time was used. As for the analytical method the column was pre-equilibrated with 3.4 mM sodium acetate, 10% ethanol and 1% diethylene glycol, adjusted to pH 4.4 (buffer A). After loading the PEG-IFN samples, the column was washed with buffer A, followed by an ascending linear gradient to 10 mM dibasic potassium phosphate, 10% ethanol and 1% diethylene glycol, adjusted to pH 6.6 (buffer B). The flow rate was 1.0 mL/min and the detection at 218 nm.

[0044] The protein concentration of the PEG-IFN alpha 2a isomer was determined by spectrophotometry, based on the 280 nm absorption of the protein moiety of the PEG-IFN alpha 2a.

[0045] An analytical elution profile of 180 µg of PEG-IFN alpha 2a is shown in Fig.1. The result of this method is a separation into 8 peaks, 2 peaks with baseline separation and 6 with partial separation. The decrease of the baseline absorption towards the end of the chromatogram suggests that there were no other monopegylated species of IFN alpha 2a eluting at higher retention time.

[0046] In addition, looking carefully at the IEC-chromatogram a further peak close to the detection limit is visible between peaks 2 and 3 indicating the presence of additional positional isomers that should also contribute to the specific activity of the PEG-IFN alpha 2a mixture. Additional species were expected as the interferon alpha-2a molecule exhibits 12 sites for pegylation (11 lysines and the N-terminus). However, given the low abundance of these species, they were not isolated and characterised.

[0047] Isomer samples derived from IEC optimisation runs were investigated directly after the isolation ($t = 0$) and after 2 weeks of storage at 5°C (data not shown). No significant differences were observed for the protein derived from IEC-peaks with regard to the protein content as determined by spectrometric methods; nor were any changes to be detected in the monopegylation site, the content of oligo-PEG-IFN alpha 2a, the amount of aggregates and the bioassay activity. Taking into account the relative abundance of the individual isomers - as determined by the IEC method - as well as the specific activities - as determined in the anti-viral assay – almost the total specific bioactivity of the PEG-IFN alpha 2a mixture used for their isolation is recovered (approximately 93%).

[0048] The analytical IE-HPLC was used to check the purity of the individual isomers with respect to contamination with other positional isomers in the IEC fractions. The peaks 2, 3, 4, 4a, 5 and 7 had more than 98%, the peaks 1 and 8 had 93% and peak 6 had 88 % purity.

Table 1:

[0049] PEG-peptides identified by comparison of the Lys-C digest spectra of the isomers and the reference standard.

Identified PEG Sites in the separated PEG-IFN Species

Peak		missing peaks in peptide map	
PEG-IFN	PEG site	M _r (DA)	Sequence
Peak 1	K ³¹	A, E	24-49
Peak 2	K ¹³⁴	I, I'	134-164
Peak 3	K ¹³¹	C	122-131 ^a
Peak 4	K ¹²¹	B, C	113-131
Peak 4a	K ¹⁶⁴	b	134-164 ^{a,b}
Peak 5	K ⁷⁰	D, F	50-83
Peak 6	K ⁸³	D, H	71-112
Peak 7	K ⁴⁹	E, F	32-70
Peak 8	K ¹¹²	B, H	84-121

^a 132-133 too small to detect.

^{a,b} RP-HPLC.

[0050] The fractions were characterised by the methods described in examples 2 to 6.

Example 1B

Analytical separation of positional isomers of mono-pegylated interferon alpha 2a

[0051] HPLC Equipment: HP1100

Column: SP-NPR, TosoH Bioscience, Particle size: 2.5µm, nonporous,

Order#: 13076

Injection: 5-10 µg monopegylated IFN

mobile Phase: *Buffer A:*

10% v/v Ethanol

1% v/v Diethylenglycol

2.3 mM Na-Acetate

5.2 mM Acetic acid, in purified water, no pH adjustment

Buffer B:

10% v/v	Ethanol
1% v/v	Diethylenglycol
16.4 mM	KH ₂ PO ₄
4.4 mM	K ₂ HPO ₄ , in purified water, no pH adjustment

[0052] Gradient: 0 Min 40 %B

2 Min 40 %B

2.1 Min 48 %B

25 Min 68 %B

27 Min 75 %B

30 Min 75 %B

34 Min 40 %B

40 Min 40 %B

Flow: 1.0 ml/min

Column Temperature: 25°C

Detection: 218 nm

a typical Chromatogram is given i Figure 8.

Example 2

Analysis of the fractions by mass spectrometry peptide mapping

[0053] Mass spectra were recorded on a MALDI-TOF MS instrument (PerSeptive Biosystems Voyager-DE STR with delayed extraction). Each IEC fraction (Ion Exchange Chromatography) was desalted by dialysis, reduced with 0.02 M 1,4-dithio-DL-threitol (DTT) and alkylated with 0.2 M 4-vinyl pyridine. Then the proteins were digested with endoproteinase Lys-C (Wako Biochemicals) in 0.25 M Tris (tris(hydroxymethyl)-aminoethane) at pH 8.5 with an approximate enzyme to protein ratio of 1:30. The reaction was carried out over night at 37 °C.

[0054] A solution of 20 mg/ml α -cyano-4-hydroxycinnamic acid and 12 mg/ml nitrocellulose in acetone/isopropanol 40/60 (v/v) was used as matrix (thick-layer application). First, 0.5 μ L of matrix was placed on the target and allowed to dry. Then, 1.0 μ L of sample was added. The spectra were obtained in linear positive ionisation mode with an accelerating voltage of 20.000 V and a grid voltage of 95 %. At least 190 laser shots covering the complete spot were

accumulated for each spectrum. Des-Arg¹-bradykinin and bovine insulin were used for internal calibration.

Example 3

high-performance liquid chromatography (RP-HPLC) Peptide Mapping

[0055] The peptides were characterized by reverse-phase high-performance liquid chromatography (RP-HPLC) Peptide Mapping. The IEC fractions were reduced, alkylated and digested with endoproteinase Lys-C as described for the MALDI-TOF MS peptide mapping. The analysis of the digested isomers was carried out on a Waters Alliance HPLC system with a Vydac RP-C18 analytical column (5 μ m, 2.1 \times 250 mm) and a precolumn with the same packing material. Elution was performed with an acetonitrile gradient from 1 % to 95 % for 105 min in water with a flow rate of 0.2 mL/min. Both solvents contained 0.1 % (v/v) TFA. 100 μ L of each digested sample were injected and monitored at 215 nm.

Example 4

MALDI-TOF spectra of undigested protein

[0056] An 18 mg/ml solution of trans-3-indoleacrylic acid in acetonitrile/0.1 % trifluoroacetic acid 70/30 (v/v) was premixed with the same volume of sample solution. Then 1.0 μ L of the mixture was applied to the target surface. Typically 150 - 200 laser shots were averaged in linear positive ionisation mode. The accelerating voltage was set to 25.000 V and the grid voltage to 90 %. Bovine albumin M⁺ and M²⁺ were used for external calibration.

Example 5

SE-HPLC (size exclusion HPLC)

[0057] SE-HPLC was performed with a Waters Alliance 2690 HPLC system equipped with a TosoHaas TSK gel G 4000 SWXL column (7.8 \times 300 mm). Proteins were eluted using a mobile phase containing 0.02 M NaH₂PO₄, 0.15 M NaCl, 1 % (v/v) diethylene glycol and 10 % (v/v) ethanol (pH 6.8) at a flow rate of 0.4 mL/min and detected at 210 nm. The injection amounts were 20 μ g of each isomers.

[0058] Size Exclusion HPLC and SDS-PAGE were used to determine the amount of oligo-PEG-IFN alpha 2a forms and aggregates in the different IEC fractions. The reference material contains 2.3 % aggregates and 2.2 % oligomers (Fig. 4).

[0059] Peaks 1, 4, 4a, 5, 6 and 8 contain < 0.7 % of the oligopegylated IFN alpha 2a forms, whereas in peaks 2, 3, and 7 the percentage of the oligopegylated IFN alpha 2a forms are under the detection limit (< 0.2 %). In the case of the aggregates a different trend could be seen. In all peaks the amount of aggregates is below 0.9 %.

Example 6

SDS-PAGE

[0060] SDS-PAGE was carried out both under non-reducing and under reducing conditions using Tris-Glycine gels of 16 % (1.5 mm, 10 well). Novex Mark 12 molecular weight markers with a mass range from 2.5 to 200 kDa were used for calibration, bovine serum albumin (BSA) was used as sensitivity standard (2 ng). Approximately 1 µg of all the samples and 0.5 µg of standard were applied to the gel. The running conditions were 125 V and 6 W for 120 min. The proteins were fixed and stained using the silver staining kit SilverXpress from Novex. The gels that were recorded under non-reducing conditions for the IEC fractions 1 - 8 (Fig. 2) show a pattern that is comparable to that of the PEG-IFN alpha 2a reference standard.

[0061] Under reducing conditions, the gels show an increase in intensity of the minor bands at about 90 kDa as compared to the standard. Between 6 and 10 kDa protein fragments appear for peaks 6, 7 and 8 (Fig. 3). Both bands together correspond to approximately 1 % of clipped material. In the lanes of isomer 1, 5, 6, 7, 8 additional bands with more than 100 kDa can be seen which are also present in the standard. These can be assigned to oligomers. Thus SDS-PAGE confirms the results of the SE-HPLC analysis.

[0062] Overall, RP-HPLC and SDS-PAGE experiments indicate that the purity of the IEC fractions can be considered comparable to the PEG-IFN alpha 2a reference standard. The structure of the PEG-IFN alpha 2a species derived from the 9 IEC-fractions were identified based on the results of the methods described above using the strategy mentioned above.

Example 7
The antiviral activity (AVA)

[0063] The antiviral activity was estimated by its protective effect on Madin-Darby bovine kidney (MDBK) cells against the infection by vesicular stomatitis virus (VSV) and compared with a PEG-IFN alpha 2a standard. Samples and reference standard were diluted in Eagle's Minimum Essential Medium (MEM) containing 10 % fetal bovine serum to a final concentration of 10 ng/mL (assay starting concentration). Each sample was assayed in quadruplicate.

[0064] The antiviral protection of Madin-Darby bovine kidney cells (MDBK) with vesicular stomatitis virus was tested according to the method described in *Viol.* 1981, 37, 755-758. All isomers induced an activity in the anti-viral assay as presented in Table 2. The activities range between 1061 and 339 U/ μ g, indicating that the difference in specific activities of the protein in the positional isomers is significant. The know-how and the results generated so far will allow the initiation of further investigations to establish this structure-function relationship between the positional isomers and the IFN alpha receptors.

Table 2:

[0065] In Vitro Antiviral Activities of PEG-IFN alpha 2a and individual PEG-IFN alpha 2a isomers. The Antiviral activity was determined in MDBK cells infected with vesicular stomatitis virus. The results present the averages of three assays performed independently.

Antiviral Assay of PEG-IFN	
Peak	U/ μ g
PEG-IFN	1061 \pm 50
Peak 1	1818 \pm 127
Peak 2	1358 \pm 46
Peak 3	761 \pm 97
Peak 4	339 \pm 33
Peak 4a	966 \pm 107
Peak 5	600 \pm 27
Peak 6	463 \pm 25
Peak 7	513 \pm 20
Peak 8	468 \pm 23

[0066] The results are further illustrated by the following figures

Figure 1:

[0067] Analytical IEC-HPLC of 180 μ g of PEG-IFN alpha 2a. An analytical strong-cation exchange column was used to check the purity of the separated positional isomers from each purification step (TOSOH-BIOSEP, SP-5PW, 10 μ m particle size, 7.5 mm diameter, 7.5 cm length). The peaks were collected for analysis.

Figure 2:

[0068] A/B: SDS-PAGE analysis with Tris-glycine (16%), the samples were electrophoresed under non-reduced conditions. The gels were stained for protein with Silver Stain. Lanes: M, molecular weight marker proteins/ 2, Peak 1/ 3, Peak 2/ 4, Peak 3/ 5, Peak 4/ 6, Peak 4a/ 7, Peak 5/ 8, Peak 6/ 9, Peak 7/ 10, Peak 8/ 11, 1x PEG-IFN standard/ 12, 1.5x PEG-IFN standard/ C₁, IFN standard.

Figure 3:

[0069] A/B: SDS-PAGE analysis with Tris-glycine (16%), the samples were electrophoresed under reduced conditions. The gels were stained for protein with Silver Stain. Lanes: M, molecular weight marker proteins/ 2, Peak 1/ 3, Peak 2/ 4, Peak 3/ 5, Peak 4/ 6, Peak 4a/ 7, Peak 5/ 8, Peak 6/ 9, Peak 7/ 10, Peak 8/ 11, 1x PEG-IFN standard/ 12, 1.5x PEG-IFN standard/ C₁, IFN standard.

Figure 4:

[0070] Size Exclusion (SE-) HPLC was used to determine the amount of oligo PEG-IFN forms and aggregates in the different IEC fractions. SE-HPLC was performed with a TosoHaas TSK gel G 4000 SWXL column (7.8 × 300 mm).

Figure 5:

[0071] MALDI-TOF spectrometry was used to determine the molecular weight of each isomer in order to ensure that the PEG-IFN molecules were still intact after IEC chromatography and to confirm the monopegylation.

Figure 6:

[0072] MALDI-TOF Lys-C peptide maps of the PEG-IFN reference standard and the peaks 1, 2, 3, 4, 4a, 5, 6, 7, 8. Missing peaks compared to the standard are indicated by arrows.

Figure 7:

[0073] RP-HPLC chromatograms of the Lys-C digests of the Peg-IFN reference and peak 4a

Figure 8:

[0074] Analytical HPLC of 5-10µg of PEG-IFN alpha 2a mixture of positional isomers on a column charged with SP-NPR, TosoH Bioscience, Particle size: 2.5µm, nonporous as described in Example 1B.

Figure 9:

[0075] Ribbon structure of interferon alpha-2a showing the pegylation sites. This is the high resolution structure of human interferon alpha-2a determined with NMR spectroscopy see *J. Mol. Biol.* 1997, 274, 661-675. The pegylation sites of pegylated interferon alpha-2a are coloured red and labelled with residue type and residue number.